Characterization of L1400

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Project: Amoco CRADA
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Objective:
To investigate the effect of temperature, aeration and the initial glucose level on cell mass production and ethanol yield with the yeast *Saccharomyces diastaricus* (L1400, supplied by Amoco).

Background:
L1400 is the parent strain to the recombinant xylose fermenting organism, LNH35, developed by Dr. Nancy Ho at Purdue University funded by Amoco/DOE. LNH35 is a candidate for the production of ethanol from biomass due to its ability to co-ferment xylose and glucose which is a necessary characteristic form economical process.

Growth and ethanol production studies were carried out with the parent strain, L1400, because it is the first phase organism under the Amoco CRADA to be used in the PDU prior to the recombinant strain LNH35. It is also hoped that information gained with L1400 will be transferable to LNH35.

Materials and Methods:
Nine batch fermentations were performed to investigate the effect of the three parameters, temperature. aeration, and initial glucose level. on ethanol. cell mass and by-product production. The experiments were designed using the computer software package Design-Ease in a full factorial fashion. Table 1 summarizes the parameters for each fermentation in randomized order.

Run (#)	Temperature (°C)	Aeration (vvm)	Initial glucose (g/L)
1	30	0.5	50.0
2	37	0.5	20.0
3	37	0.0	20.0
4	33.5	0.25	35.0
5	30	0.5	20.0
6	30	0.0	20.0
7	37	0.5	50.0
8	37	0.0	50.0
9	30	0.0	50.0

Table 1: Experimental Parameters

Inoculum preparation:
The inoculum for each batch run was prepared in two stages. In the first stage 1 ml from a frozen stock vial of L1400 was inoculated into an erlenmeyer flask containing 50 mls of YPD (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose, pH 5). The first stage was incubated for 12 hours and then 10% v/v was inoculated into a second flask consisting of 1% w/v CSL and 2% w/v glucose (pH 5). Each stage was incubated at the temperature of the fermentation run with an agitation of 150 rpm. A 10% v/v inoculum was transferred from stage 2 into the fermentation vessel after 12 hours of incubation.

Fermentation Conditions:
The nine experiments were performed in batch mode employing New Brunswick BIOFLO III fermentors with a 1 L working volume. The medium for each fermentation was 1% w/v CSL (not filtered) and the appropriate glucose concentration depending on the run (see Table 1) at pH 5. Three runs were performed at one time with the pH controlled at 5 with 3M NaOH, agitation at 150 rpm and the temperature set at the appropriate run temperature (see Table 1). House air or nitrogen, depending on the run (see Table 1) was filtered through .2 µm and sparged into the fermentors at 0.25 or 0.5 vvm. The exhaust gas went through a condenser that was chilled with house cold water.

Sampling protocol:
Ethanol and glucose concentrations were monitored throughout each run by Yellow Springs Instrument (YSI), and subsequently by HPLC. Growth was measured by optical density (OD) measurements (600 nm) and *dry* cell weight. To obtain *dry* cell weights. a 5 ml sample was centrifuged, washed twice and dried at 60°C for 24 hours. The pH of the fermentations was monitored for each run by checking it on an externally calibrated pH electrode and adjusting the BIOFLO reading as necessary. Major by-products were also identified and quantified from the HPLC samples.

Results and Discussion:
Figures 1 and 2 depict the effect of temperature, aeration. and initial glucose level on cell production as determined by OD measurements.

Figure 1

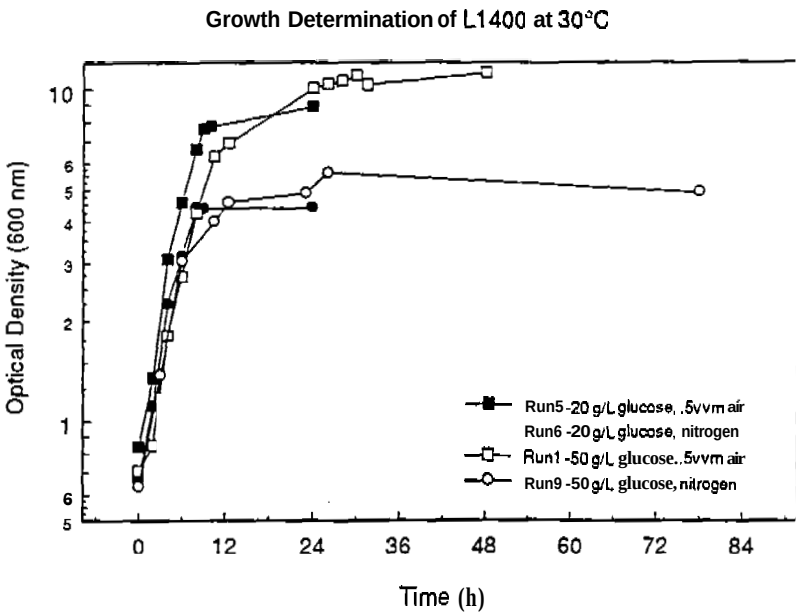
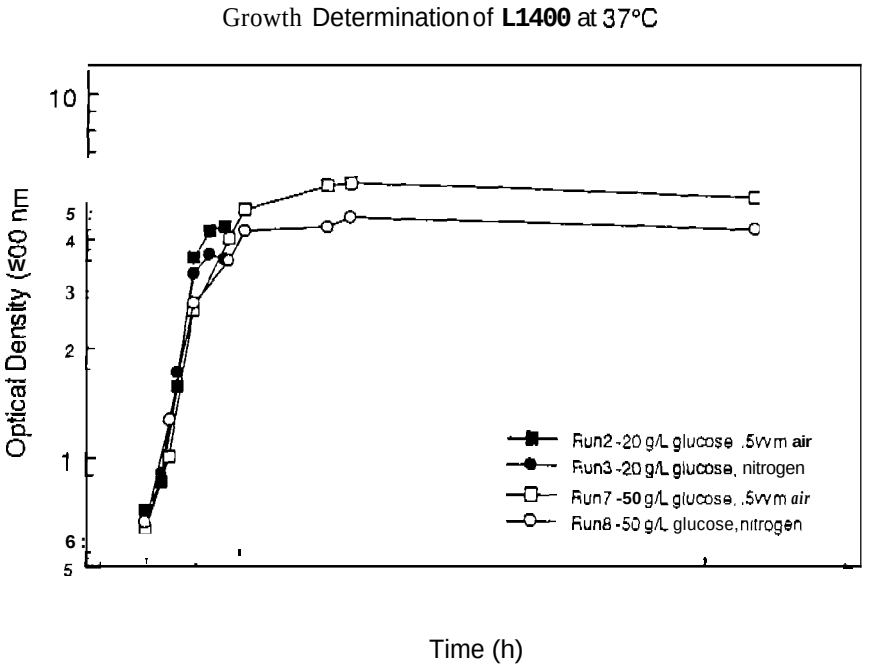


Figure 2



A higher final cell mass **and** growth rate is observed with aeration. In addition. the lower temperature (30°C) favors cell mass production. **A** statistical analysis **was** performed on the data which is summarized in figure 8. This figure shows the statistical effect of all three factors on cell mass yield, growth rate and the maximum *dry* cell weights obtained. On **this** graph. the different bars represent the normalized effects (i.e., effects divided **by** their standard deviation) of the various components. **A** positive effect for a component signifies an increase in the response under consideration (e.g.. ethanol) caused by the addition of the component; the opposite holds for a negative effect. The *two* horizontal dashed lines denote the 95% significance level. Bars higher than these lines represent significant effect. This graph clearly depicts the positive effect of aeration on all three responses **as** well as the negative effect of a high initial glucose (50 g/L) on cell mass yields and growth rates. It also shows a slight negative effect of temperature on cell mass yield.

Unlike cell mass yield, aeration does not seem to have an effect on ethanol yield, **and** the higher temperature (37°C) resulted in **an** increase in ethanol production. ***Akin*** to cell mass yield, a higher **initial** glucose concentration of 50 g/L decreases the ethanol yield and production rate. The following two graphs (figs. 3 **and 4)** show the effect of all three parameters on ethanol production. The ethanol yield was calculated based on the highest ethanol concentration obtained. The graph shows that the ethanol concentration declines after reaching **a** maximum. This may be due to evaporation and subsequent loss of ethanol through the condenser.

Figure 3

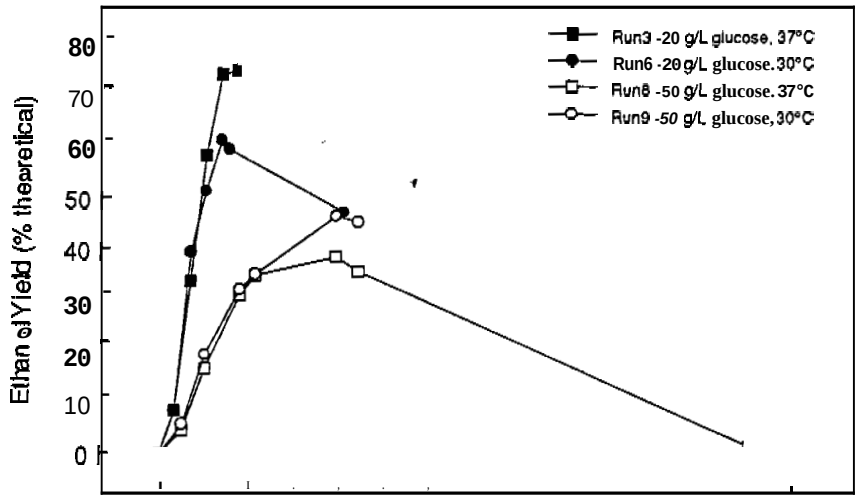
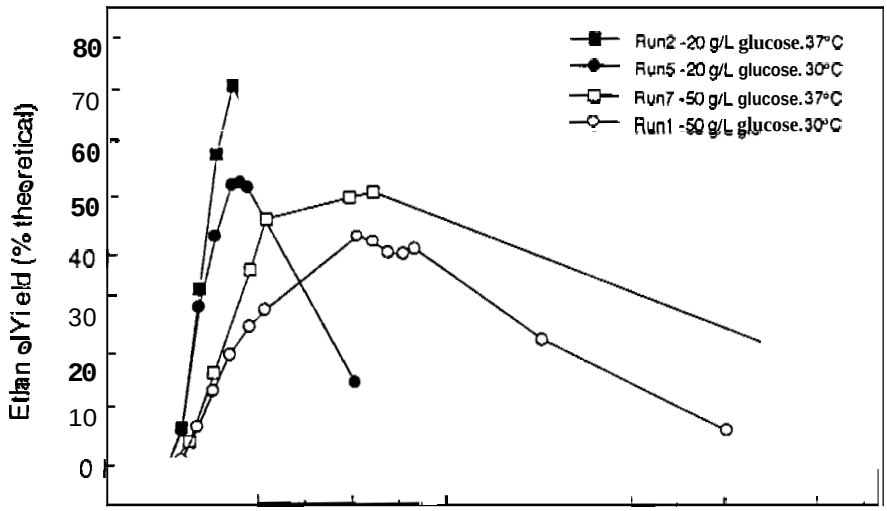


Figure 4



Once again a statistical analysis was performed on the data (see **fig.** 9) **and** it shows the relevance of three factors on ethanol yield, ethanol production rate, **and** the final ethanol concentration achieved. As the raw data suggests, the plot demonstrates the negative effect of glucose on ethanol yield and production rate and the positive effect of temperature on the ethanol production rate.

By-products:

Unlike the yeast *Saccharomyces cervesiae*, D5A, where very little if any by-product formation is observed, strain L 1400 readily forms **by-products.** The major by-product produced by L1400 is glycerol (.03 - .16 g glycerol/ g glucose) representing a potential significant shuttle of carbon away **from** ethanol production. Smaller amounts of lactic acid **and** acetic acid are produced with negligible amounts of succinic acid production. Figures ***5*** and 6 show the concentrations of glycerol produced during each run. Since lactic acid **and** acetic acid levels are so small, they are not represented graphically.

Figure *5*

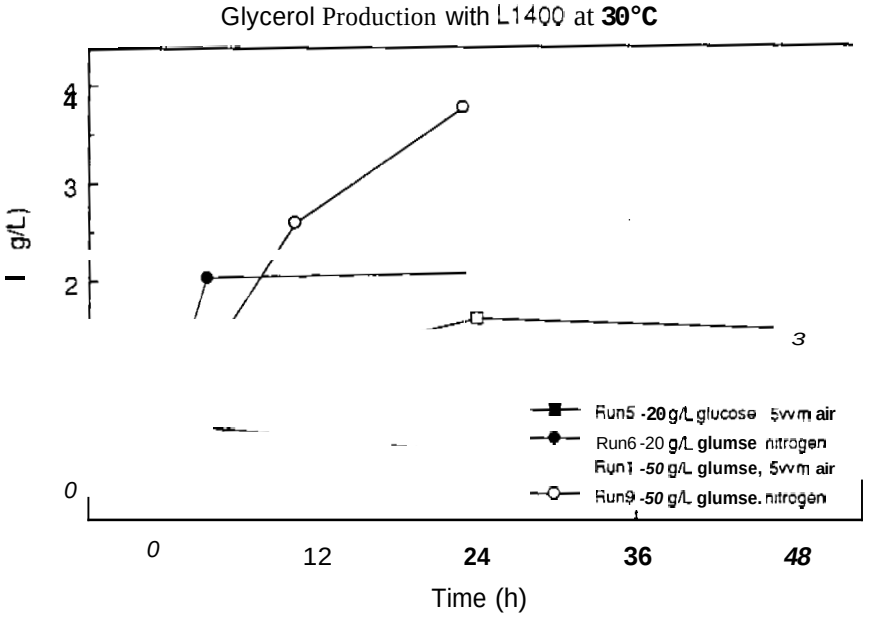
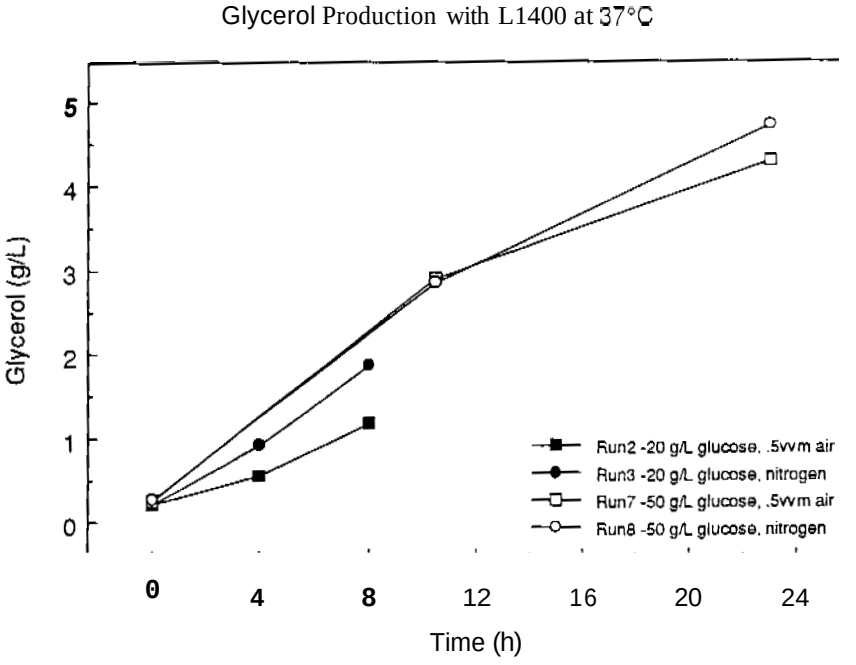


Figure 6



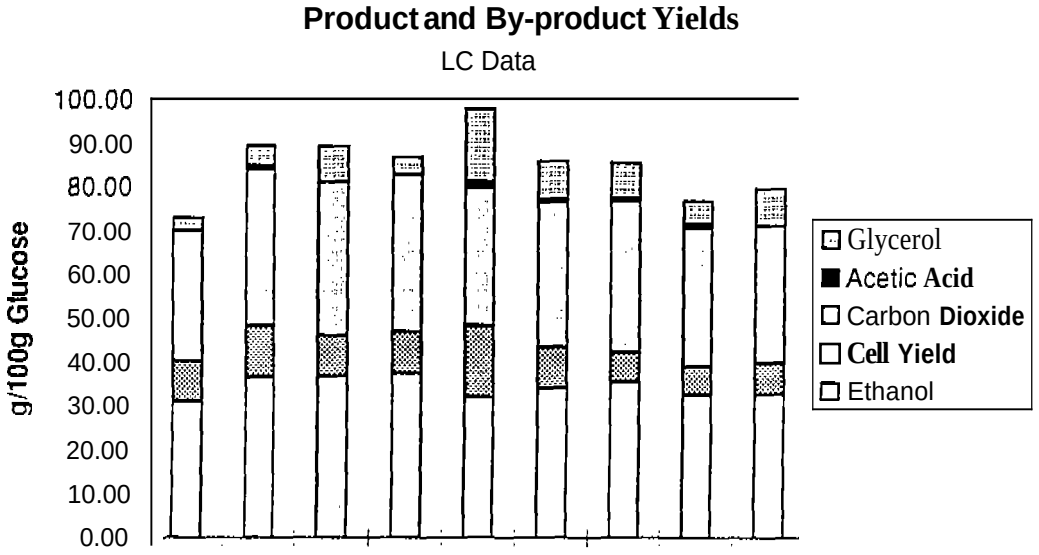
Unfortunately the temperature that favors ethanol production also favors glycerol production demonstrated by an increased yield of glycerol at 37°C compared to 30°C. Unlike ethanol production where aeration has little if any effect on yield, anaerobic conditions favor glycerol production. However, aerobic conditions seem to favor acetic acid production. In addition, the higher initial level of 50 g/L glucose also increases glycerol production. It is interesting to note that the parameters that yield the greatest amount of glycerol are opposite from those parameters that favor cell mass production. The statistical analysis plot show this point clearly (see fig. 10).

In addition to obtaining cell mass, ethanol, and by-product yields. a carbon balance was performed for each of the nine runs. This information shows the distribution of carbon under various operating conditions and helps us *quantify* carbon lost to contamination in the ethanol Process Development Unit (PDU). Carbon dioxide was calculated based on ethanol production were 1 mole CO_2 is produced per mole of ethanol. Good carbon balance closure was obtained for the low initial glucose level (20 g/L) fermentations. Unfortunately the higher initial level of glucose (50 g/L) runs had a lower percent closure (see table 2 and fig. 7). This may be due to evaporative loss of ethanol through the condenser that was nor measured. Future runs of this nature should employ the mass spec capability of the laboratory in order to obtain better carbon balance closures and ethanol yields.

Run Number	Growth Rate (h^{-1})	ETOH Yield (g/100g C)	Cell Yield (g/100g C)	Acetic Acid (g/100g C)	Glycerol (g/100g C)	CO_2 (g/100g C)	Total (g/100g C)
1	0.763	31.14	9.12	0.00	4.41	29.79	74.47
2	0.355	36.55	11.68	0.88	4.48	35.61	88.56
3	0.317	36.79	9.14	0.00	7.98	35.19	89.10
4	0.36	37.45	9.43	0.00	4.12	35.82	86.82
5	0.326	31.92	16.31	1.44	2.06	31.58	52.26
6	0.303	33.96	9.36	0.66	5.76	32.97	85.23
7	0.309	35.44	6.77	0.71	8.05	34.41	84.56
8	0.232	32.41	6.46	0.78	5.45	31.57	76.09
9	0.261	32.55	7.26	0.00	8.42	31.14	79.38

Table 2: Carbon Balance **Summary**

Figure 7



Summary:
Maximum ethanol yield **and** cell mass yield were obtained with the lower **initial** glucose concentration of 20 g/L versus the high level of *50* g/L. However. cell mass yield was greater at 30°C and ethanol yield was greater at 37°C. Aeration increased cell mass yield whereas it had no effect on ethanol yield.

The PDU consists on two separate operations; one for the production of inoculum (seed train) **and** a second for the fermentation of substrate to ethanol. Based on the above results. we are now able to maximize either cell mass yield for the seed train or ethanol yield in the fermentations and minimize glycerol production depending on the desired product. For the production of cell mass in the seed train. it is preferable to maximize the cell mass yield over ethanol production. To **do** this. the fermentation should be aerated **and** the temperature lowered to 30°C. Since **increasing** the **initial** level of glucose seems to have a deleterious effect to the rate of cell mass production. this process wouldn't be of benefit in **trying** to increase the cell mass yield. However. **high** glucose does increase the final cell mass concentration. Therefore, the trade off between the time factor versus the desired final cell mass yield should be determined in order to optimize cell **mass** yield in the seed train.

In the fermentation, the production of ethanol is of **primary** importance. Unlike seed production, the temperature should **be** raised to 37°C. The higher temperature is better for the performance of the enzyme as well. Since aeration has no effect on ethanol production, and it is costly to supply, the fermentation should not be aerated, especially at the large scale of 9000L. However, these conditions will also favor glycerol production. One would have to determine the economics of aerating the fermentations to decrease glycerol production versus the amount of ethanol that is lost **from** glucose being shuttled away from ethanol production to glycerol production.

Figure 8

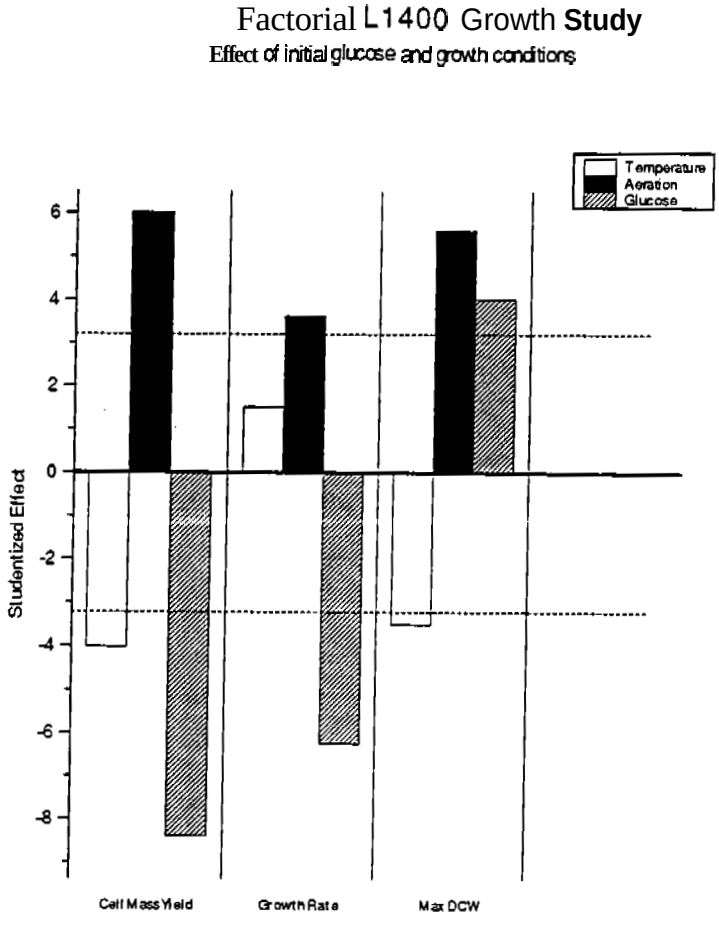

Factorial L1400 Growth Study
Effect of initial glucose and growth conditions
Temperature
Aeration
Glucose
Studentized Effect
6
4
2
0
-2
-4
-6
-8
Cell Mass Yield
Growth Rate
Max OCW

Figure 9

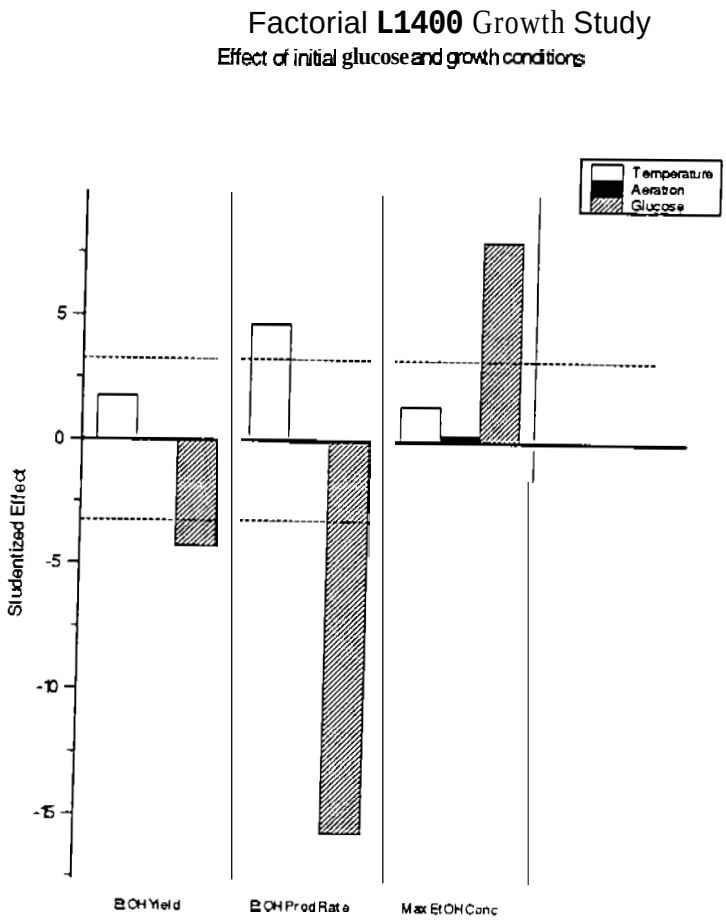
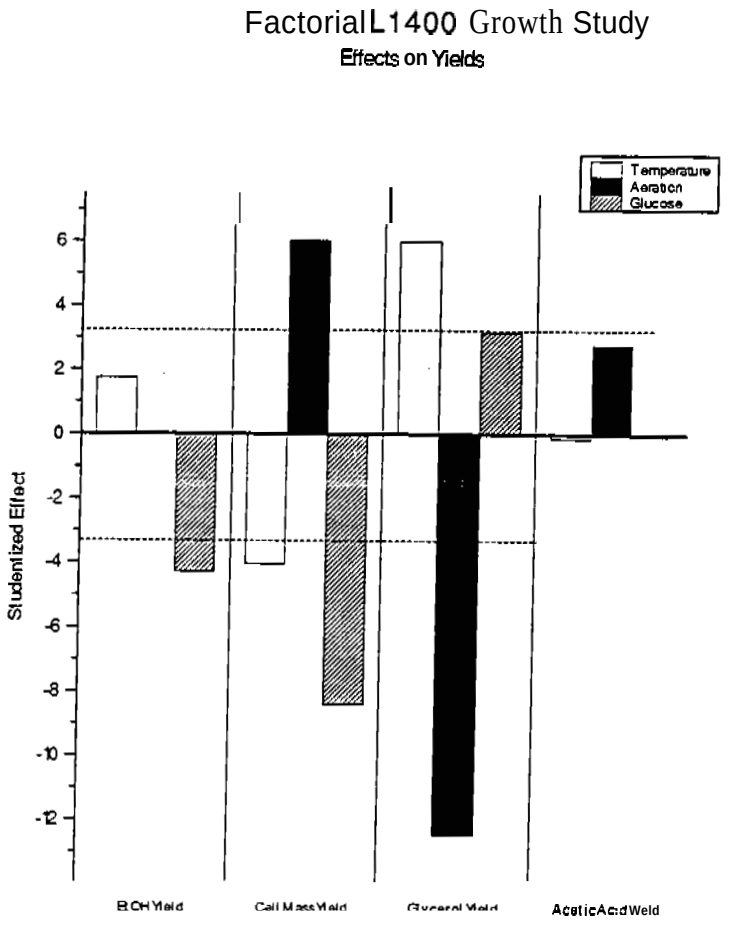

Factorial L1400 Growth Study
Effect of initial glucose and growth conditions
Temperature
Aeration
Glucose
Studentized Effect
5
0
-5
-10
-15
EtOH Yield
EtOH Prod Rate
Max EtOH Conc

Figure 10


Factorial L1400 Growth Study
Effects on Yields
Temperature
Aeration
Glucose
Studentized Effect
6
4
2
0
-2
-4
-6
-8
-10
-12
EtOH Yield
Cell Mass Yield
Glucose Yield
Acetic Acid Yield

Raw Data From Nine Batch Fermentations

?UN1

.140(30°C, 0.5 vm, 50g/L nitial glucose, pH 5.0

		YSI		LC				DCW
Time (h)	OD (600 nm)	**glucose** (g/L)	ethanol (g/L)	**glucose** (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
0	0.708	**54**	0.95	51.9	1.1037	0.00	0.00	0.66
2	0.844	51.4	1.45					
4	1.a	45.8	3.18					
6	2.72	42.2	5.1					
8	4.24	35.4	7					2.88
10.5	6.27	28.4	8.45					
12.5	6.91	24.3	9.31	24.341a	9.6866	0.00	**1.22**	3.17
24	10.06	6.56	**13.21**	5.9346	12.6263	**0.29**	1.60	5.44
26	10.38	4.38	**12.94**					
28	10.58	2.51	12.38					
30	11	0.874	**12.29**					
31.5	10.32	0.113	12.57					6
48	11.2	0.0019	7.87	0	7.7761	0.79	1.47	
72		0.0035	3.12					

		YSI		**LC**				
Time (h)	OD (600 nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	**Acetic** Acid (g/L)	Glycerol (g/L)	DGW (g/L)
0	0.713	21.3	1.02	21.0581	1.1756	0	0.2112	0.5
2	0.852	20.7	1.88					
4	1.56	**12**	4.75	12.0929	4,5133	0	**0.5426**	
6	3.53	4.66	7.53					
8	4.18	0	8.95	0	8.8731	0.1846	1.1556	2.96
10	4.3	0	8.3					

RUN 3

L1400, 37°C, 0.00 vvm (sparged with nitrogen), 20g/L initial glucose, pH 5.0

		YSI		LC				
Time (h)	OD (600 nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
0	0.633	20.2	1.05	20.5577	1.2236	0	0.2115	0.56
2	0.899	17.97	1.95					
4	1.7	11.8	4.57	11.8907	4.7251	0	0.9039	
6	3.2	5.33	7.11					
8	3.6	0.002	8.77	0	8.7866	0	1.8517	2.44
10	3.48	0	8.84					

RUN 4

		YSI		LC				
Time (h)	OD (600nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
0	0.741	36.2	1.28	39.5246	1.1017	0	0.1978	0.54
2	1.375	33.6	2.42					
4	2.52	23.6	7.38					
6	4.87	13.2	10.42					
8	6.56	3.75	14.24	4.1179	14.3612	0	1.6569	3.88
9	6.95	0.052	15					4.28
10	7.16	0.0065	15.1					
24	8.15	0.003	12.72	0	13.3415	0.457	1.3065	

RUN 5

		YSI		LC				
Time (h)	OD (600 nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW
0	0.834	19.95	1.22	20.5297	1.0392	0	0.1934	0.66
2	1.345	17.84	1.98					
4	3.07	12.05	4.37					
6	4.56	6.38	5.73					
8	6.6	1.98	6.73	2.2581	6.8712	0.2625	0.5698	3.64
9	7.59	0.423	6.79					4.02
10	7.73	0.017	6.69					
24	8.86	0.004	2.92	0.0045	3.1526	1.2239	0.2802	

RUN 6

				glucose, pH 5.0				
				LC				
				glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
				21.4567	1.1283	0	0.1975	0.56
8	4.41	0.267	8.17	0.526	8.2368	0.139	2.0301	2.52
9	4.37	0.005	7.98					2.42
24	4.39	0.005	6.6	0	7.2448	0	2.0683	

RUN 7

140C 37°C, 0.5 vm air,50.0 g/L initial gl

		YSI		LC				
Time (h)	OD (600nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
0	0.63	58.13	1.1	52.6222	I.1488	0	0.2508	0.54
3	1	49.38	2.66					
6	2.53	38.1	6.54					
10.5	3.98	19.5	12.35	19.8098	12.7768	0.2325	2.8933	2.76
12.5	4.79	13.4	15.23					
23	5.6	0.021	16.48	0	17.7635	0.3642	4.2853	3.52
26	5.7	0.027	16.79					
78	5.21	0.0035	8.17					

RUN 8

140C 37°C, 0.0 vvm air,50.0 g/L initial glucose. pH 5.0

		YSI		LC				
Time (h)	OD (600 nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
0	0.658	59.13	1.19	51.9871	1.2036	0	1.2036	0.52
3	1.26	49.5	2.66					
6	2.64	34.7	6.33					
10.5	3.46	21.25	10.64	21.9437	10.9401	0.2347	2.8397	2.46
12.5	4.18	17.6	11.83					
23	4.3	0.343	12.97	0.398	12.8122	0.342	4.7125	3.08
26	4.58	0.035	12.09					
78	4.24	0.0095	1.84					

RUN 9

		YSI		LC				
Time (h)	OD (600nm)	glucose (g/L)	ethanol (g/L)	glucose (g/L)	ethanol (g/L)	Acetic Acid (g/L)	Glycerol (g/L)	DCW (g/L)
0	0.632	57.25	1.14	52.0747	1.6214	0	0.2908	0.48
3	1.37	48.75	2.91					
6	3.03	34.3	6.87					
10.5	3.98	24.35	10.62	24.8163	10.4947	0	2.5866	2.46
12.5	4.57	21.05	11.52					
23	4.86	4.15	14.9	4.5527	14.7296	0.1358	3.7532	3.1
26	5.58	1.16	14.56					
78	4.88	0.004	1.34					